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PATENT

CELLULAR VACCINES AND IMMUNOTHERAPEUTICS AND METHODS FOR THEIR PREPARATION

RELATED APPLICATION

The present application claims the priority benefits of U.S. provisional application 60/019,639, filed June 12, 1996.

FIELD OF THE INVENTION

The present invention provides a method for enhancing the immunogenicity of weakly immunogenic or non-immunogenic cells in order to provide the immune system with an immunogenic signal capable of stimulating T cell activation leading to an effective immune response. The method of the invention generates cellular vaccines which are useful for the prevention and treatment of diseases which develop and/or persist by escaping the immune response triggered by T cell activation. Such diseases include, for example, all cancers, natural and induced immune-deficiency states, and diseases caused by infections with a variety of pathogens.

BACKGROUND OF THE INVENTION

U.S. patent 5,484,596 by Hanna et al. describes using tumor tissue as a vaccine. U.S. Patent 4,844,893 by Honsik et al. describes arming IL-2-activated leukocytes with Mabs directed to antigens preferentially expressed on tumor cells for killing the target cells. Both patents are incorporated by reference herein.

Anti-tumor immune responses are mediated primarily by T

lymphocytes. Down regulation of both the major histocompatibility complex (MHC) and the molecules that costimulate the immune response is associated with defective T cells activation signaling by tumor cells (Luboldt et al., Cancer Res. 56:826-830, 1996; L. Chen et al., 1992, Cell 1: 1093; P.S. Linsley, J. A. Ledbetter, 1993, Ann. Rev. Immunol 11: 191; G.J. Freeman et al., 1993, Science 262: 909; C.H. June et al., 1994, Immune. Today 15: 321; J. T. Gerge et al., 1993, Cancer Res. 53: 2374; Ostrand-Rosenberg, 1993, S. Curr. Opin. Immune. 6: 772; B. E. Elliot et al., 1989, Adv. Cancer Res. 53: 181).

T cell receptor (TCR) recognition of MHC-bound antigen is not a sufficient signal for T cell activation. Costimualtory molecules, such as B7-1 and B7-2, are cell surface proteins of antigen presenting cells (APCs), and other cells targeted by the immune response, that provide critical signals for T cell activation (for review, see L. Chen et al., 1995, Immunol. Rev. 145: 123; T. Tykocinski et al., 1996, Am. J. Path. 148: 1). B7 signaling via the T cell surface molecule CD28 appears to be the major costimulatory pathway for T cell activation. However, recent studies show that costimulation is a more complex event which involves both cytokines and adhesion molecules (G. Yang et al., 1995, J. Immune. 154: 2794; M. Kubin et al., 1994, J. Exp. Med. 180: 211; Y. Li et al., 1996, J. Exp. Med. 183: 639).

Many approaches have been used to enhance the immunogenicity of tumor cells (see, for example, the references cited in this section). The major approaches presently under investigation involve gene transfer. In this regard, most of the methods

employed to date have required ex vivo or in vivo transfection with genes such as MHC or B7, or modification of tumor cells with antigen presenting cells (APCs) (Y. J. Guo et al., 1994, Science 263: 518; M. Tykocinski, 1996, A. J. Path. 148: 1; J. Young and K. Inaba, 1996, J. Exp. Med. 183: 7; L. Zitvogel et al., 1996, J. Exp. Med. 183: 87; C. M. Celluzzi et al., 1996, J. Exp. Med. 183: 283). These approaches are time consuming and problematic because of the poor transfectability of primary tumor cells and because of the requirement for large numbers of APCs.

In vitro treatment of tumor cells with cytokines increases the expression of MHC and adhesion molecules (R. Mattsson et al., 1992, Biol-Reprod 46: 1176; R. J. Ulevitch et al., 1991, Am. J. Pathol. 139: 287; F. Willems et al., 1994, Eur. J. Immune. 24: 1007; I. Saito et al., 1993, J. Clin. Lab. Anal. 7: 180; R. A. Panettieri et al., 1994, J. Immune. 154: 1358; M. Ikeda et al., 1994, J. Invest. Dermatol. 103: 791). Transfection of tumor cells with MHC, B7-1 and B7-2 genes converts low immunogenic tumor cell lines to immunogenic cell lines (S. E. Townsend and J. P. Allison, Science 259: 368; J. P. Allison et al., 1995, Curr. Opin. Immune. 7: 682; G. Yang et al., 1995, J. Immune. 154: 2794; M. Kubin et al., 1994, J. Exp. Med. 180: 211). Non-immunogenic tumor cells are not responsive to transfection with the B7 gene alone but can become responsive by co-expression of CD48 molecules at the cell surface (Y. Li et al., 1996, J. Exp. Med. 183: 639).

The costimulatory molecule B7 can under some circumstances deliver a negative signal through its binding to CTLA-4, a second

receptor for B7 on T cells. Cross-linking CTLA-4 molecules in vitro has been shown to inhibit T cell proliferation. Furthermore, mice deficient in CTLA-4 develop severe T cell proliferative disorders (K. Kawai et al., 1993, Science 261: 609; J. P. Allison, M. K. Krummel, 1995, Science 270: 932; J.M. Green et al., 1994, Immunity 1: 501). A recent report showed that the introduction of anti-CTLA-4 monoclonal antibody (MAb), which blocks CTLA-4 mediated signaling, resulted in enhanced T cell-dependent rejection of tumors in certain mouse models (D.R. Leach et al., 1996, Science 271: 1734). These data provide evidence that CTLA-4 may be counter-regulatory to the CD28 costimulatory signal. Thus, transfected tumor cells expressing B7 molecules may fail to elicit effective immunity due to CTLA-4 mediated negative signaling.

In addition to T cell activation using B7 gene transfection, bispecific monoclonal antibodies (Bi-MAbs) in combination with pre-stimulated lymphocytes have been used to induce T cell activation under certain circumstances. For example, one study reports that costimulatory signals can be delivered by a combination of Bi-MAbs to CD28:CD30 (CD30 is a Hodgkin's tumor-associated antigen) and CD3:CD30 in combination with peripheral blood lymphocytes (PBLs) pre-stimulated with the CD3:CD30 Bi-MAb in the presence of CD30+ Hodgkin's tumor-derived cells; however, the combination of CD28:CD30 and CD3:CD30 Bi-MAbs alone did not induce significant in vitro cytotoxicity of resting human PBLs against a Hodgkin's tumor-derived cell line, and stimulation with the CD28:CD30 Bi-MAb alone was not effective (C. Renner et al.,

1994, Science 264: 833). Similarly, regression of Hodgkin's derived tumor xenografts was observed only when both the CD28:CD30 and CD3:CD30 Bi-MAbs were used in combination with PBLs prestimulated in vitro with CD30+ cells and CD3:CD30 Bi-MAb; no significant effect was observed in xenografts treated with either of the Bi-MAbs alone, or a combination of the two Bi-MAbs without prestimulated human PBLs (Renner et al., supra).

SUMMARY OF THE INVENTION

The present invention features immunogenic tumor cells and other immunogenic autologous cells, convenient methods of making such immunogenic cells, methods of using such immunogenic cells to activate or enhance immune response against diseased cells with minimum effect on normal or healthy cells, and methods of avoiding the negative T cell signaling pathway.

The present invention provides a method for enhancing the immunogenicity of weakly-immunogenic or non-immunogenic cells, resulting in a cellular vaccine that can stimulate T cell activation, which in turn leads to an effective immune response against diseased cells. The cellular vaccines of the present invention can be used as vaccines to prevent diseases and as immunotherapeutics to treat diseases.

In summary, the method of the invention involves the steps of (1) treating weakly- or non-immunogenic autologous cells (target cells) in order to amplify primary and costimulatory T cell activation signals in the cells, and (2) attaching to the treated cells a substance capable of binding to one or more

antigens on the treated cells and to one or more T cell activation costimulatory molecules on the surface of T cells (such as CD28), thereby providing the treated cells with the capacity to physically link to T cells and to activate the costimulatory signal. Such substances include, but are not limited to, bispecific monoclonal antibodies (Bi-MAbs) targeted to antigen on the treated cells and to CD28 and/or other costimulatory molecules on T cells. The first step may be skipped when the autologous cell is attached with (1) a bridge molecule with two or more binding sites for T cell activation costimulatory molecules on the surface of T cells, or (2) two or more bridge molecules each with one or more binding sites for T cell activation costimulatory molecules on the surface of T cells.

Once the primary and/or costimulatory T cell activation signals in the target diseased cells have been amplified by cytokines or other means and the bridge molecules have been attached to the target diseased cells, the cytokines and the bridge molecules not attached to the target diseased cells may be removed from the immunogenic composition before the target diseased cells are administered to a patient. This additional step minimizes adverse effects associated with administering cytokines to a patient. It also minimizes the risk associated with allowing bridge molecules not attached to a target diseased cell into a patient, an event which may cause unwanted immune response against normal or healthy cells.

The first step of the method up-regulates antigen processing

capacity within the treated cells and amplifies the expression of cell surface molecules involved in T cell activation. The second step provides the treated cells with a means to physically bridge to T cells via CD28 and/or other costimulatory molecules, thereby providing optimal conditions for stimulating T cell activation.

Thus, in a first aspect, this invention features an immunogenic composition for administration to a patient mammal (including a human) having target diseased cells. The immunogenic composition contains an autologous target diseased cell which differs from the diseased cells in the patient in that it processes and presents antigens characteristic of the diseased cells more effectively. For example, the autologous target diseased cell expresses one or more primary (e.g., MHC) and/or costimulatory (e.g., B7-1 and B7-2) T cell activation molecules at a higher level (e.g., 50% higher, preferably 2 folds higher, more preferably 10 folds higher). As described below, there are different ways of enhancing the expression level of the primary and/or costimulatory T cell activation molecules.

In addition, the autologous target diseased cell has attached thereto one or more bridge molecules. Each bridge molecule has one or more binding sites for one or more costimulatory molecules on the surface of effector cells, which include, but are not limited to, T cells, NK cells, macrophages, LAK cells, B cells, and other white blood cells. Preferably, though not required, the bridge molecules have one or more binding sites for one or more antigens on the surface of the target diseased cell and are attached to the target diseased

cells at the cell surface antigens. In another preferred embodiment, substantially all (e.g., >80%, preferably >90%, more preferably >95%) the bridge molecules in the immunogenic composition are attached to the autologous target diseased cells so that the composition is substantially free of bridge molecules not attached to a target diseased cell. In a further preferred embodiment, the immunogenic composition contains a pharmaceutically effective amount of the target diseased cells with bridge molecules attached thereto.

By "immunogenic" is meant the ability to activate the response of the whole or part of the immune system of a mammal, especially the response of T cells.

By "autologous" is meant that the target diseased cell is from the patient mammal, or from another patient having a common major histocompatibility phenotype. An autologous target cell may be obtained from the patient mammal or another source sharing the same MHC with methods known to those skilled in the art.

By "target diseased cell" is meant a cell causing, propagating, aggravating or contributing to a disease in a patient mammal. Target diseased cells include, but are not limited to, tumor cells (including unmodified tumor cells, tumor cells modified with different approaches, and primary culture). The sources of tumor cells include, but are not limited to, liver cancer, hepatocellular carcinoma, lung cancer, gastric cancer, colorectal carcinoma, renal carcinoma, head and neck cancers, sarcoma, lymphoma, leukemia, brain tumors, osterosarcoma, blade carcinoma, myloma, melanoma, breast cancer, prostate cancer,

ovarian cancer, and pancreas carcinoma.

Target diseased cells may also be cells infected with prions (which cause Mad Cow diseases among others), viruses, bacteria, fungi, protozoa or other parasites (e.g. worms).

Viruses include those described or referred to in Fields Virology, Second Edition, 1990, Raven Press, New York, incorporated by reference herein. Examples include, but are not limited to, herpes virus, rhinoviruses, hepatitis virus (type A, B, C and D), HIV, EBV, HPV, and HLV.

Bacteria include those described or referred to in Bergey's Manual of Determinative Bacteriology, Ninth Edition, 1994, Williams and Wilkins, incorporated by reference herein. Examples include, but are not limited to, gram positive and negative bacteria, *streptococci*, *pseudomonas* and *enterococci*, *Mycobacterium tuberculosis*, *Aeromonas hydrophilia*, *Aeromonas caviae*, *Aeromonas sobria*, *Streptococcus uberis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Bacillus sphaericus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Serratia liquefaciens*, *Lactococcus lactis*, *Xanthomonas maltophilia*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Streptococcus constellatus*, *Streptococcus anginosus*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, and *Klebsiella pneumonia*.

Primary T cell activation molecules include MHC class I, MHC class II and other molecules associated with antigen processing and/or presentation. Costimulatory T cell activation molecules include ICAM-1, ICAM-2, ICAM-3, LFA-1, LFA-2, VLA-1, VCAM-1, 4-1-BB, B7-1, B7-2, and other cell adhesion proteins and other cell

surface proteins which can activate T cell costimulatory pathways through T cell surface proteins.

By "bridge molecule" is meant a molecule or substance which can bring two or more cells together by attaching to the cells with its binding sites. Preferably, a bridge molecule can bring an autologous target diseased cell together with an effector cell and deliver a signal to the effector cell to activate or enhance the effector cell's immune response against the target. A bridge molecule has one or more binding sites for stimulatory and/or costimulatory molecules on the effector cells. These binding sites can be designed to activate a positive regulator of T cell activation (e.g., CD28, 4-1BB) but avoid stimulating a negative regulator of T cell activation (e.g., CTLA-4). The binding sites can also be designed to blockade a negative regulator of T cell activation (see Leach et al., Science 271:1734-1736, 1996). A bridge molecule may also have one or more binding sites for antigens on the surface of the target diseased cell. Bridge molecules include, but are not limited to, bispecific monoclonal antibodies, fusion proteins, organic polymers, and hybrids of chemical and biochemical materials. The antibodies described or disclosed in U.S. patents 5,601,819, 5,637,481, 5,635,602, 5,635,600, 5,591,828, 5,292,668 and 5,582,996 are incorporated by reference herein.

The antigen on the target cell serving as an anchor for the bridge molecule need not be unique to the target cell when the bridge molecule is attached to the target cell *in vitro*. Any molecule on the target cell surface can be used to anchor the

bridge molecule, including, but not limited to, proteins, glycoproteins, lipids, glycolipids, phospholipids, lipid aggregates, steroids, and carbohydrate groups such as disaccharides, oligosaccharides and polysaccharides (see "Molecular Biology of The Cell," pp47-58, pp276-337, Second Edition, published by Garland Publishing, Inc. NY & London). Examples include transferrin receptor, Low Density Lipoprotein (LDL) receptor, gp55, gp95, gp115, gp210, CD44, ICAM-1, ICAM-2, collagen and fibronectin receptor, transferrin receptors, Fc receptor, and cytokine receptors.

Costimulatory molecules on the surface of effector cells may be antigens, fatty acids, lipids, steroids and sugars that can stimulate or costimulate these effector cells' functions to destroy the target cells. Costimulatory molecules include, but are not limited to, CD1a, CD1b, CD1c, CD2, CD2R, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CDw12, CD13, CD14, CD15, CD15s, CD16a, CD16b, CDw17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32, CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a, CD42b, CD42c, CD42d, CD43, CD44, CD44R, CD45, CD45RA, CD45RB, CD45RO, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, CD51/61 complex, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CDw60, CD61, CD62E, CD62L, CD62P, CD63, CD64, CDw65, CD66a, CD66b, CD66c, CD66d, CD66e, CD67, CD68, CD69, CD70, CD71, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD80, CD81, CD82, CD83, CDw84, CD85, CD86, CD87, CD88, CD89, CDw90, CD91, CDw92, CD93, CD94, CD95, CD96, CD97, CD98, CD99, CD99R, CD100, CDw101,

CD102, CD103, CD104, CD105, CD106, CD107a, CD107b, CDw108, CDw109, CD110-CD114, CD115, CDw116, CD117, CD118*, CD119, CD120a, CD120b, CDw121a, CDw121b, CD122, CD123*, CDw124, CD125*, CD126, CDw127, CDw128, CD129, CDw130, LFA-1, LFA-2, LFA-3, VLA-1,VCAM-1, VCAM-2, 4-1BB, cytokine and chemokin receptors. In a preferred embodiment, the bridge molecule has a binding site for CD28 or 4-1BB on the surface of T cells.

By "pharmaceutically effective" is meant the ability to cure, reduce or prevent one or more clinical symptoms caused by or associated with the diseased cells in the patient mammal, including, but not limited to, uncontrolled cell proliferation, bacteria infection, and virus infection.

The immunogenic composition may be isolated, enriched or purified for administration to a patient.

By "isolated" in reference to the immunogenic composition is meant that the autologous target diseased cell is isolated from a natural source. Use of the term "isolated" indicates that one or more naturally occurring materials have been removed from the normal environment. Thus, the target diseased cell may be placed in a different cellular environment or in a solution free of other cells. The term does not imply that the target diseased cell is the only cell present, but does indicate that it is the predominate cell present (at least 20 - 50% more than any other cells) and is essentially free (about 90 % pure at least) of other tissues naturally associated with it in the body of the patient. In a preferred embodiment, the composition is substantially free of effector cells such as T cells. In another

preferred embodiment, the composition is substantially free of bridge molecules not attached to a target diseased cell. In a third preferred embodiment, the composition is substantially free of cytokines outside of the target diseased cell.

By "enriched" in reference to the immunogenic composition is meant that the autologous target diseased cell constitutes a significantly higher fraction (2 - 5 fold) of the total cells in the composition than in the diseased tissue in the patient's body. This could be caused by a person by preferential reduction in the amount of other cells present, or by a preferential increase in the amount of the specific target diseased cells, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other cells present, just that the relative amount of the cell of interest has been significantly increased in a useful manner. The term "significantly" here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other cells of about at least 2 fold, more preferably at least 5 to 10 fold or even more.

By "purified" in reference to the immunogenic composition does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the target diseased cell is relatively purer than in the natural environment. The target diseased cells could be obtained directly from the patient or from cell culture, with or without modifications. Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five

orders of magnitude is expressly contemplated. In a preferred embodiment, the composition is substantially free of effector cells such as T cells.

The immunogenic composition may contain a pharmaceutically suitable carrier or excipient. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). The immunogenic composition may be administered to a patient systemically, e.g., by intravenous infusion or subcutaneous injection. A composition of the invention may be administered as a unit dose to a patient mammal, each unit containing a predetermined quantity (e.g., about $1x10^5$ to about $1x10^{10}$, preferably about $1x10^6$ to about $1x10^9$, and more preferably about $1x10^7$ to about $1x10^8$) of armed and/or activated autologous target diseased cells calculated to produce the desired therapeutic effect in association with the physiologically tolerable aqueous medium as diluent.

The expression of primary and costimulatory T cell activation molecules may be enhanced by various means, for example, *in vitro, ex vivo* or *in vivo* treatment of target cells with cytokines or other factors capable of inducing the desired amplification; and *in vitro* and *in vivo* transfer to the target cells of MHC genes, adhesion molecule genes, cytokine genes, and/or their respective transcription activators or enhancers. Cytokines include those described or referred to in The Cytokine Handbook, Thomson, A., (ed.), 1994, Academic Press, San Diego, incorporated by reference herein. Examples include, but are not

limited to, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, inteferons (e.g., IFN α, β, and γ), tumor necrosis factors (e.g., TNF α, and β) and other chemokines and lymphokines. In a preferred embodiment, IFN γ and TNF α are used either alone or in combination to enhance the expression of primary and costimulatory T cell activation molecules in autologous target diseased cells.

When a target diseased cell coated with bridge molecules is administered into a patient, it will bind to costimulatory molecules on the surface of the effector cells. The more densely the target diseased cell is coated with bridge molecules, the more effector cells it will be able to bind. In addition, the more binding sites a bridge molecule has for the costimulatory molecules, the more effector cells it will be able to bind.

In that regard, Applicant has found that a cellular vaccine may be prepared without the need of cytokine treatment (to increase the levels of primary and costimulatory T cell activation molecules) when a plurality of bridge molecules are attached to a target cell with binding sites for two or more different costimulatory molecules on the surface of T cells. Individual bridge molecules may be attached to different anchor molecules on the surface of the target diseased cell. An individual bridge molecule may also have two or more binding sites for two or more different costimulatory molecules on the surface of T cells.

Thus, in a second aspect, this invention features an

immunogenic composition containing an autologous target diseased cell having attached thereto (a) a bridge molecule which has two or more binding sites for two or more different effector cells, (b) a bridge molecule which has two or more binding sites for two or more different costimulatory molecules on the surface of effector cells, (c) two or more bridge molecules each containing a binding site for a different effector cell, (d) two or more bridge molecules each containing a binding site for a different costimulatory molecule on the surface of effector cells, (d) two or more bridge molecules each attached to a different antigen on the target cells, or (e) a combination of two or more of the above.

A pharmaceutically effective amount of an immunogenic composition of this invention may be complemented by a pharmaceutically acceptable carrier before administration to a patient mammal.

Alternatively, a patient may be administered with a pharmaceutical composition containing (1) a pharmaceutically effective amount of a cytokine capable of increasing the level of one or more primary and costimulatory T cell activation molecules in tumor cells, (2) a pharmaceutically effective amount of a bridge molecule containing a binding site for an antigen on the surface of the tumor cells and a binding site for a costimulatory molecules on the surface of T cells, and (3) a pharmaceutically acceptable carrier.

In treating a patient, the autologous target cell may be treated with cytokines or other means of increasing primary and

costimulatory T cell activation molecules *in vitro* before the target cell is administered to the patient. Alternatively, the cytokines may be administered to the patient to increase primary and costimulatory T cell activation molecules *in vivo*.

In a third aspect, this invention features a method of generating cytotoxic leukocytes against diseased cells in a patient mammal by contacting a population of effector cells (e.g., white blood cells) *in vitro* with immunogenic compositions described above for a time period sufficient to react with the immunogenic compositions and collecting the treated effector cell population. The cytotoxic leukocytes so generated can then be administered to a patient to treat or prevent diseases.

The method of the invention is useful for the prevention and treatment of diseases which develop and/or persist by escaping immune responses triggered by T cell activation. Such diseases include, for example, all cancers, natural and induced immune deficiency states, and diseases caused by infections with a variety of pathogens. The method of the invention is illustrated herein by demonstrating its application to three different types of human cancers. Cancer cells are by nature generally weakly immunogenic, fail to trigger an effective T cell response, and survive and grow as a result. As demonstrated herein, cancers can be prevented, and established cancers may be cured, by stimulating an effective T cell response using autologous tumor cell vaccines of the invention.

Other features and advantages of the invention will be apparent from the following detailed description of the

invention, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Expression of MHC class I, ICAM-1, ICAM-2 and VCAM-1 antigens on cytokine treated hepa 1-6 cells. The results are representative data from four comparable experiments.

FIG. 2. Stimulation and proliferation of syngeneic splenic T cells in vitro induced by cytokine treated hepa 1-6 cells and anti-CD28 MAb.

FIG. 3. Cytotoxicity of CTLs generated by in vitro priming of naive splenic T cells with cytokine treated hepa 1-6 cells in combination with anti-CD28 Bi-MAbs or control MAb.

FIG. 4. Induction of protective immunity with cytokine treated hepa 1-6 cells armed with anti-CD28 Bi-MAb.

FIG. 5. Tumor rejection following treatment with cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb.

FIG. 6. Cure of established hepatomas in vivo.

FIG. 7. Therapeutic effectiveness of γ-irradiated cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for immunizing individuals against disease and for treating individuals with established diseases using cellular vaccines created with a two-step process described herein. The methods of the invention may be applicable to any disorder involving a low- or non-immunogenic response pathology, wherein effective treatment or prophylaxis

requires an immune boost through activation of T cells. Such disorders include, but are not limited to, all forms of cancer, immune deficiency disorders (both natural and induced), and infectious diseases caused by viral or other pathogenic agents.

The methods of the invention comprise modifying weakly- or non-immunogenic autologous cells of the disorder (target cells) by (1) treating the target cells in order to amplify primary and costimulatory T cell activation signals therein, and (2) attaching to the target cells a substance capable of binding to one or more antigens on the target cells and to one or more T cell activation costimulatory molecules on the surface of T cells (i.e., CD28), thereby providing the target cells with the capacity to physically link to T cells and activate the costimulatory signal. Such substances include, but are not limited to, bispecific monoclonal antibodies (Bi-MAbs) targeted to antigen on the treated cells and to CD28 and/or other costimulatory molecules on T cells.

The first step of the method amplifies the expression of cell surface molecules involved in T cell activation, such as MHC and adhesion molecules, and up-regulates antigen processing capacity within the target cells by enhancing enzyme activity involved in intracellular antigen processing. For the first step of the method, any means which can amplify primary and costimulatory T cell activation signals in the target cells (i.e., the expression of MHC and adhesion molecules), may be used. Such amplified expression may be achieved by, for example, in vitro and in vivo treatment of target cells with cytokines or

other factors capable of inducing the desired amplification; and in vitro and in vivo transfer of MHC genes, adhesion molecule genes, cytokine genes, and/or MHC, adhesion molecule, and cytokine gene transcription activators or enhancers to the target cells.

In one embodiment of the method, amplification of primary and costimulatory T cell activation signals in the target cells is achieved using cytokine treatment. Target cells may be treated with cytokines ex vivo or in vitro as described in the examples herein. Alternatively, cytokines may be administered to the target cells in vivo by, for example, intralesional injection, intralymph injection, subcutaneous injection, etc., in suitable pharmaceutical carriers or controlled release preparations. Any cytokine or combination of cytokines which results in the amplified expression of MHC and adhesion molecules may be used to treat cells in the first step of the method. In preferred embodiments, described more fully by way of the examples herein, a combination of interferon (IFN-γ) and tumor necrosis factor-α (TNF-α) is used in the first step. Preferably, cells may be treated with concentrations of between about 10 - 100 U IFN-γ in combination with concentrations of between about 10-100 U TNF-α, more preferably with 100 U IFN-γ, and 50 U TNF-α, as described in Section 6.1., infra. However, the conditions and specific cytokines most optimal for the amplification of activation signals on the particular cells to be treated may vary and may be determined essentially as described in Section 6.1., infra.

The second step of the method of the invention provides the treated cells with the capacity to physically bridge to T cell surfaces via CD28 and/or other T cell costimulatory molecules, thereby providing optimal conditions for stimulating T cell activation. For the second step, any substance capable of binding to one or more antigens on the treated cells and to one or more T cell activation costimulatory molecules on the surface of T cells may be used. Such bispecific or multispecific bridging substances may comprise, for example, Bi-MAbs, proteins and other macromolecules, and polymer materials, which contain a functionality capable of binding to the targeted T cell costimulatory molecule and activating, or inducing the activation of, the costimulatory signal. In one embodiment, described by way of the examples in Section 6, infra, Bi-MAbs are used as the bridging substance.

One functionality of the bispecific or multispecific bridging substance may be directed to a target cell-specific antigen or any antigen expressed on the target cells. Optimally, where the target cells are to be armed with bridging substance in vivo, the target cell antigen to which the bridging substance is directed should be unique.

However, the target cell antigen need not be unique to the treated cells, since the attachment of the bridging substance may be practiced *in vitro*. Accordingly, bridging substances attached to the target cells *in vitro* will not cross-react with the same antigen on cells in the individual to be immunized with the modified cells.

After such bridging substances are incubated with cells treated according to the first step of the method, free bridging substance may be washed away and bound bridging substance may be cross-linked to the cell surface with polyethylene glycol (PEG) or another cross-linking agent.

Another functionality of the bispecific or multispecific bridging substance is specifically directed to a T cell activation costimulator such as CD28. Thus, when the modified cells are used to immunize an individual, the CD28 (or other costimulatory molecule) binding sites of the attached bridging substance are free, and will bind to CD28 (or other costimulatory molecule) on T cell surfaces, ensuring that the modified cells will become physically linked to T cells. This bridging substance-mediated physical link also brings other molecules on the surfaces of the modified cells, some of which have been amplified by the cytokine treatment step, into contact with other molecules on the surfaces of T cells, providing further costimulation which thereby further facilitates T cell activation.

The second step of the method may be practiced in vitro or in vivo, depending upon whether target cells were treated according to the first step of the method in vivo or in vitro, the circumstances of the disease or lesion to be treated, and the clinical objectives of the treatment. Where the first step of the method is conducted in vivo, the treated cells may be armed with the bridging substance in vivo as well. In this case, the clinician may use a variety of known methods for administering

the bridging substance to a patient. The best route of administering the bridging substance to patients who have had disease- or lesion- specific cells (target cells) treated in vivo according to the first step of the method will depend on clinical and/or other aspects of the disease or lesion to be treated as well as on the site of the treated cells. For example, where target cells located in lymph node have been treated in vivo, direct administration of the bridging substance to the lymph node is preferred. Similarly, for example, where tumor cells have been treated in vivo by intratumor injection of cytokines or gene transfer vectors, the bridging substance preferably should be administered directly into the tumor or to the local environment of the tumor.

Where the first step of the method is conducted in vitro, the treated cells may be armed with the bridging substance in vivo or in vitro. Where the bridging substance is administered in vivo, the same route used to administer the treated cells or a similar route should be used, taking into account the same factors discussed above regarding in vivo arming of in vivo treated target cells.

When in vitro treated cells are armed in vitro, the treated and armed cells (cellular vaccine) may be used in vivo for treatment and prevention of disease, or in vitro for generation of lesion- or disease- specific cytotoxic T lymphocytes (CTLs). Arming treated cells in vitro provides the advantage of being able to use a bridging directed to any antigen on the target cell.

When used for treatment or immunization of a patient, in vitro treated and armed cells may be administered to the patient using a variety of methods known to those skilled in the art. In a particular embodiment, described more fully in the examples which follow, in vitro treated and armed cells are administered subcutaneously. In another embodiment, the treated and armed cells are administered by direct intralesion injection, an administration route that may provide advantages over subcutaneous administration in certain circumstances (for example, where the lesion to be treated is not well vascularized, is inaccessible for biopsy, or cannot be disrupted without creating further risk to the patient). In a further embodiment, the treated and armed cells are administered by injection into the lymph nodes. This method of administration requires fewer treated and armed cells than may typically be required using other routes of administration. Such intralymph administration may be preferred in situations where only limited autologous tissue can be obtained from a lesion using thin needle biopsy techniques (i.e., inaccessible/inoperable cancers). Moreover, intralymph administration is likely to enhance the interaction between the cellular vaccine and T cells given the large number of T cells within lymph nodes. Applicant's initial experimental data indicates that a single intralymph injection of as few as 1 X 10^4 cellular cancer vaccine cells, prepared using the method of the invention, can induce an effective immune response against parenteral tumor cell challenge and can cure established tumors. The therapeutic efficacy of this method of administration appears

equivalent to that achieved using 100-times more cells administered subcutaneously.

In a specific embodiment of the method of the invention, Bi-MAbs that react with CD28 are used as a bispecific bridging substance. As briefly discussed in Section 2, supra, B7 interacts with both CD28 and CTLA-4 on T cells. Under certain circumstances, the B7-CTLA-4 interaction generates a negative signal which prevents T cell activation. Thus, by immunizing with cells coated with Bi-MAbs specific for CD28, the interactions between B7 on such cells and CTLA-4 (or other T cell activation down-regulating molecules) is minimized and/or by-passed. In addition, Bi-MAbs reactive with other costimulatory T cell surface molecules (i.e., CD2, CD48) may also be used in the practice of the method of the invention. Furthermore, more powerful costimulation may be achieved by using a multiplicity of Bi-MAbs having specificity for various T cell costimulators.

Bridging substances may be prepared using well known technologies. As shown in the examples which follow, Bi-MAbs may be used effectively as the bridging substance. Such Bi-MAbs may be generated using methods well known in the art, such as, for example, those described in Section 6.2., infra, and as described in the references cited therein. Bi-MAbs containing multiple T cell costimulatory molecule binding sites may be prepared by chemical linkage in order to provide a means for generating multiple costimulatory activation circuits.

In addition to Bi-MAbs, molecules engineered to contain functional binding sites specific for both the antigen(s) of the

target cell(s) and the T cell costimulatory molecule(s) may be used as the bridging substance in the practice of the method of the invention. Such molecules may, for example, comprise proteins, other macromolecules, and polymers engineered to contain the desired binding sites, and may be prepared by using genetic engineering technologies, synthetic technologies, or by chemical linkage of component polypeptides, polymers, and/or other macromolecules. The binding site components of such bridging substances may comprise, for example, Fab2 antibody fragments, antibody binding sites, natural or engineered ligands, or other factors reactive with the target cell antigen(s) and T cell costimulatory molecule(s).

The bridging substance may be administered to a patient in vivo using a pharmaceutically acceptable carrier or a variety of drug delivery systems well known in the art. As an example, for cancer immunotherapy, a combination of the cytokines TNF-α and IFN-γ and an anti-CD28 Bi-MAb may be formulated within a controlled release preparation which is administered to the patient by directly injecting the preparation into the lymph nodes or into the tumor itself.

The method of the invention is particularly useful in treating cancers. This aspect of the invention is more fully described by way of the examples presented in Section 6., infra. The data presented in the examples indicate that strongly immunogenic tumor cells can be generated using the two-step method of the invention, comprising (1) in vitro treatment of autologous tumor cells with a combination of γ- interferon (IFN-

γ) and tumor necrosis factor-α (TNF-α), and (2) pre-incubation with a Bi-MAb specific for both antigen on tumor cells and CD28 on T cells. The resulting modified tumor cells are able to act as a cellular vaccine that elicits CTL-mediated immunity which can both prevent and cure established tumors.

In particular, the studies described in the examples which follow show that cytokine-treated, anti-CD28 Bi-MAb-armed hepatoma cells induce protective immunity against parental tumor cell challenge and, moreover, cure established gross hepatomas in mice. In addition, the studies described in Section 6.7., infra, show that the method of the invention also induces protective immunity against lymphoma and colon carcinoma.

Different routes of administration, or combinations thereof, may be preferred when treating different cancers or other diseases using the cellular vaccines of the invention. As illustrated by the study briefly described in Section 6.4., immunization with cytokine-treated (and un-armed) cells followed by intravenous administration of an anti-CD28 Bi-MAb induces some anti-tumor immunity in the hepatoma model system.

In comparison, as shown by the results of the studies described in Sections 6.5. and 6.6., infra, the administration of cells treated with cytokines in vitro and armed with Bi-MAbs in vitro induces uniform tumor immunity and cures established hepatomas. It is possible that insufficient localization of the Bi-MAbs to the tumor tissue following intravenous injection in the former case is responsible for the difference in therapeutic efficacy.

Individuals may be immunized against a variety of diseases with cytokine-treated, anti-CD28 Bi-MAb-armed autologous cells, thereby providing the individual's immune system with a signal sufficient to activate T cells and confer protective immunity. Similarly, individuals may be treated for a variety of diseases by administering cytokine-treated, anti-CD28 Bi-MAb-armed autologous cells of the disease or lesion, thereby providing the individual's immune system with a signal sufficient to activate T cells and induce a cytotoxic T lymphocyte response. In both cases, BI-MAbs with a specificity for other T cell costimulatory molecules may be used to arm the treated cells with a means to physically bridge to T cells in vivo.

In addition to MHC class I, ICAM-1, ICAM-2 and VCAM-1 molecules, treatment with cytokines may enhance tumor antigen processing by tumor cells, and may induce the expression of other cell surface molecules essential for T cell activation. The combination of cytokine treated hepa 1-6 cells and immobilized anti-CD28 MAb fails to stimulate splenic T cells in vitro or to induce anti-tumor immunity in vivo in applicant's model system. This suggests that the signal delivered by the interaction between CD28 and anti-CD28 MAb is not sufficient in itself to induce T cell activation.

In contrast, a strongly immunogenic response is obtained when cytokine treated tumor cells armed with anti-CD28 Bi-MAb in vitro interact with CD28 on T cells, indicating that a physical bridging function is an important component of the activation process.

In addition, the observation that cytokine treated, B7 transfected hepa 1-6 cells were not able to activate splenic T cells in vitro (Section 6.2., infra) is consistent with the recent finding that B7 may interact with CTLA-4 to deliver a negative regulatory signal, and provides a strong rationale for using anti-CD28 Bi-MAbs to physically link the antigen presenting cell specifically to CD28 molecules for T cell activation.

The invention provides an effective alternative to gene transfer and tumor:APC engineering for the development of cellular vaccines. In particular embodiments of the invention, described in the following examples, the attachment of Bi-MAbs to tumor or other target cells takes place in vitro. Accordingly, the antigens on such cells need not be unique to those cells. Essentially, any antigen may be targeted. Bi-MAbs can be produced by linking anti-CD28 MAbs to Mabs that recognize any antigen expressed on the tumor or other target cell, including antigens which are also expressed on large populations of cells in the individual to be treated (i.e., lymphocytes). This approach may be particularly useful in situations where Mabs to tumor specific antigens are not available.

EXAMPLE 6.1
CYTOKINE INDUCED EXPRESSION OF ADHESION AND MHC MOLECULES ON HEPA 1-6 CELLS IN VITRO

Hepa 1-6 is a chemically induced hepatoma originating in a C57BL/6 mouse (G. J. Darlington et al., 1980, J. Natl. Cancer Inst. 64: 809). Cells derived from this tumor grow rapidly and form subcutaneous nodules in syngeneic animals. Hepa 1-6 cells

lack both MHC class I and B7 molecules on their cell surfaces and do not induce a host immune response even when transfected with genes encoding the B7-1 or B7-2 molecule.

The conditions and cytokines most optimal for the amplification of activation signals on hepa 1-6 cells were determined as follows. One ml of Hepa 1-6 cells was plated into 24 well tissue culture plates at a concentration of 2 x 10^6 cells / ml and incubated with either IFN-γ, 100 U, or TNF-α, 50 U, or a combination of IFN-γ and TNF-α at these same concentrations in complete RPMI-1640 medium supplemented with 10 % fetal calf serum, 2mM glutamine, 1x non-essential amino acid and 1 mM sodium pyruvate for 48 hr at 37°C. Hepa 1-6 cells incubated similarly but in medium alone were used as control.

Cells were washed with phosphate-buffered saline (PBS) and stained with rat monoclonal antibodies to mouse MHC class I (M1/42), MHC class II (M5/114), CD44 (KM81) (ATCC), ICAM-1 (HA58), ICAM-2 (3C4) and VCAM-1 (51-10C9) (PharMingen, San Diego, California). To stain for mouse B7-1 (CD80) and B7-2 (CD86), we used CTLA4-Ig, a soluble fusion protein containing the variable domain of the human CTLA4 protein and the hinge, CH2, and CH3 domains of the human IgG1 constant region (Y. J. Guo et al., 1994, Science 263: 518; C. Caux et al., 1994, J. Exp. Med. 180: 1841; E. Murphy et al., 1994, J. Exp. Med. 180: 223; R. Seder et al., 1994, J. Exp. Med. 179: 299; B. Blazar et al., 1994, Blood 83: 3815; K. Hathcock et al., 1993, Science 262: 905; P Linsley et al., 1991, J. Exp. Med. 174: 561).

Cells were incubated with the antibodies or chimeric

protein for 40 minutes on ice. A rat antibody to mouse CD3 (YCD3) and a soluble human CD44-Ig chimeric protein were used as a negative controls. Cells were washed three times. Fluorescent isothiocyanate (FITC)-conjugated goat antibody to rat Ig or FITC-labeled rabbit antibody to human Ig was added for an additional 40 minutes on ice. Samples were then washed, fixed and analyzed in a FACScan (Becton Dickinson, San Jose, California). The mean fluorescent intensity in the negative control group (medium alone) was at background level except for ICAM-1.

Hepa 1-6 cells incubated with a combination of interferon (IFN-γ) and tumor necrosis factor (TNF-α) showed expression of MHC class I, intercellular adhesion molecule 2 (ICAM-2) and vascular adhesion molecule 1 (VCAM-1), and showed significantly enhanced expression of intercellular adhesion molecule 1 (ICAM-1) (FIG. 1). This pattern of expression was maintained for more than three days in vitro after removing cytokines from the medium. However, the cytokine treated hepa 1-6 cells (CT-hepa 1-6) were still able to form tumors in syngeneic animals. It was assumed that this may be because the cells were deficient in providing a CD28 mediated costimulatory signal due to absence in expression of B7.

EXAMPLE 6.2
STIMULATION AND PROLIFERATION OF SYNGENIC SPLENIC T-CELLS INDUCED BY CYTOKINE TREATED HEPA 1-6 CELLS AND ANTI-CD28 Bi-MAb IN VITRO

The following example demonstrates that cytokine activated Hepa 1-6 cells in combination with Bi-MAbs to CD28 and tumor cell

antigens stimulate proliferation of splenic T cells in vitro, indicating that such Bi-MAbs can provide a CD28 costimulatory signal. Three Bi-MAbs, CD28:gp55, CD28:gp95, and CD28:gp210, each with one binding specificity for the CD28 molecule on T cells and a second binding specificity for one of three glycoproteins expressed on tumor cell surfaces, were prepared and used as follows.

For preparation of Mabs and Bi-MAbs, Wistar rats were immunized with 2 x 10^7 Hepa 1-6 cells in CFA. Following three additional boosts with the same cells in ICFA over an 8 week period, spleen cells from immunized rats were fused with YB2/0 rat myelomas as previously described (J. Alan & T. Robin, in: Immunochemistry in Practice, Chapter 2 (Blackwell, New York, 2d ed. 1988)). More than twenty Ig-producing hybridomas were selected by immunofluorescent staining. Three antibodies reacted with hepa 1-6 cells by flow cytometry analysis. These Mabs separately recognized a 55 Kd, 95 Kd or 210 Kd glycoprotein expressed on most tumor cells as determined by immunoprecipitation. The Mabs were designated as anti-gp55, anti-gp95 and anti-gp210 respectively. Anti-mouse CD28 Mabs were generated by first immunizing Wistar rats with a mouse T cell hybridoma cell line expressing high levels of CD28 antigen on the cell surface. After cell fusion, hybridomas producing anti-CD28 MAb were selected with immunofluorscent analysis by FACScan. Anti-CD28 Mabs were further characterized and confirmed by immunoprecipitation and T cell proliferation and IL-2 production assays. Hybridoma producing anti-mouse CD18 used to generate

CD18:gp55 Bi-MAb was purchased from ATCC. All Mabs used in these experiments were purified by passage of ascites from nude mice over a protein G column.

Bi-MAbs were produced from these Mabs as previously described (J. A. MacLean et al., 1993, J. Immunol. 150: 1619; L. K. Gilliland et al., 1988, Proc. Natl. Acad. Sci. USA 85: 7719; C. Bode et al., 1989, J. Biol. Chem. 264: 944).

Normal splenic T cells were purified by nylon wool column. Purified T cells (5 x 10^6/ well) were co-cultured in complete RPMI-1640 medium at 37°C for 96 hours with 5 x 10^5 irradiated (5000 roentgens) cytokine treated (as described in Section 6.1., supra) or untreated hepa 1-6 cells in the presence or absence of CD28:gp55, CD28:gp95 or CD28:gp210 Bi-MAb. A CD18:gp55 Bi-MAb that bridges CD18 on T cells to gp55 on tumor cells and a mixture of parental CD28 plus gp55 Mabs were used as controls. Cytokine treated or untreated hepa 1-6 cells transfected with B7 gene and expressing high level of B7 molecules on cell surfaces were also used as a control. The percentage of $CD3^+CD8^+CD25^+$ T cells (mean ∇ SD) was determined by three color analysis in FACScan using Cy-Chrom™ labeled anti-CD3, PE-labeled anti-CD8 and FITC-conjugated anti-CD25 antibodies (PharMingen, San Diego, California).

Each of the Bi-MAbs was tested both in vitro and in vivo for its ability in combination with cytokine treatment of hepa 1-6 cells to activate tumor specific CTLs. Mouse splenic cells were cocultured with either cytokine treated or untreated hepa 1-6 cells in the presence of purified anti-CD28 Bi-MAb or control antibody, 50 mg/ml each, at 37°C for 9 days.

The results presented in FIG. 2 indicate that the combination of cytokine treated tumor cells and any one of the three anti-CD28 Bi-MAbs significantly stimulated splenic T cell proliferation.

No stimulation was obtained in the absence of either anti-CD28 Bi-MAbs or the cytokine treated autologous tumor cells. Interestingly, hepa 1-6 cells transfected with, and expressing high levels of B7, were not effective in stimulating naive T cells in vitro when treated with the cytokines in similar manner. The majority of lymphocytes generated by this approach were $CD3^+CD8^+CD25^+$ T cells.

EXAMPLE 6.3
IN VITRO CYTOTOXICITY OF CTLs GENERATED BY CYTOKINE TREATED HEPA 1-6 TUMOR CELLS IN COMBINATION WITH ANTI-CD28 Bi-MAbs

Cytotoxicity of CTLs generated by in vitro priming of naive splenic T cells with cytokine treated hepa 1-6 cells in combination with anti-CD28 Bi-MAbs or control MAb was established as follows. Nylon wool-enriched naive splenic T cells (5 x 10^6/well) were first stimulated in vitro by incubation with 5 x 10^5 irradiated (5000 roentgens) cytokine treated hepa 1-6 cells (as described in Section 6.1., supra) in combination with anti-CD28 Bi-MAbs, control antibodies or irradiated $B7^+$ hepa 1-6 alone at 37°C for 9 days. γ-irradiated naive splenic cells (5 x 10^6) were added into cultures as feeder cells. At the 3rd and 6th day after stimulation, 2 ml of complete RPMI-1640 medium containing recombinant human IL-2 (20 U) were added into each culture well

separately.

Cytotoxicity of CTLs toward syngeneic, allogenic tumor cells and a NK sensitive YAC-1 cell line was determined in a standard 4 hour ^{51}Cr release assay. Results from three experiments are shown in FIG. 3 as the percentage of ^{51}Cr release at 1:20 E:T ratio as measure of tumor cell lysis (mean ∇SD). These results show that CTLs generated as described herein have cytolytic activity specific to autologous tumor cells.

EXAMPLE 6.4

EFFECT OF INTRAVENOUSLY ADMINISTERED ANTI-CD28 Bi-MAbs ON TUMORIGENICITY OF CYTOKINE TREATED HEPA 1-6 CELLS

Mice injected with cytokine treated hepa 1-6 cells followed by intravenous administration of anti-CD28:gp55 Bi-MAb at a dose of 100 ug on day 1, 2 and 4, experienced delayed tumor formation and 40 percent of these mice (8/20) had tumor regression. Animals injected with a combination of parental hepa 1-6 cells and anti-CD28 Bi-MAb or a combination of CT-hepa 1-6 cells and control antibody all developed tumors and died within 60 days after the inoculation of tumor cells. Immunohistological studies at 24, 48, and 72 hr after injection of the Bi-MAbs showed that the Bi-MAbs were widely distributed in lungs, liver and kidney in addition to tumor tissue.

EXAMPLE 6.5

INDUCTION OF PROTECTIVE IMMUNITY IN VIVO WITH CYTOKINE TREATED HEPA 1-6 TUMOR CELLS ARMED WITH ANTI-CD28 Bi-MAbs

Tumor cells were first treated in vitro with a combination

of IFN-γ, 100 U, and TNF-α, 50 U, in RPMI-1640 medium with 10% fetal calf serum at 37°C, 5% CO_2 for 48 hours. Cells were then washed with Phosphate-Buffered Saline, PH 7.4 (PBS) x 3 at 20°C and incubated with anti-CD28 Bi-MAbs at a concentration of 50 ug/ml on ice for 45 min as described in Section 6.1., supra. After an additional incubation in an equal volume of 30% polyethylene glycol (PEG) in RPMI-1640 for 60 minutes at 4°C, the cells were washed x3 as described above and suspended in a final concentration of 1-2 x 10^7 / ml PBS. To arm cells with Bi-MAbs or Mabs, cytokine treated or untreated parental tumor cells were pre-incubated with respective antibodies for 45 minutes.

Five groups of C57BL/6 mice, 5 per group, were immunized subcutaneously with 1 x 10^6 cytokine treated hepa 1-6 cells armed with CD28:gp55, CD28:gp95, CD28:gp 210 or a control Bi-MAb CD18:gp55, or with 1 x 10^6 untreated hepa 1-6 cells armed with CD28:gp55 Bi-MAb. After two weeks, mice in each of the groups were challenged with 2.5 x 10^6 parental hepa 1-6 cells injected subcutaneously.

Cytokine treated hepa 1-6 tumor cells (CT-hepa 1-6) pre-incubated/armed with anti-CD28 Bi-MAbs completely lost their ability to form tumors in syngeneic mice, whereas cytokine treated hepa 1-6 cells pre-incubated/armed with control antibodies retained their tumor forming capacity. The results of the immunization experiment are shown in FIG. 4. Mice immunized with CT-hepa 1-6 cells armed with each of the CD28/tumor antigen Bi-MAbs developed protective immunity against challenge with parental tumor cells, and all of these animals remained tumor-

free for 120 days after such challenge (FIG. 4). In contrast, all mice injected subcutaneously with either untreated Hepa 1-6 cells armed with anti-CD28 Bi-MAbs or CT-hepa 1-6 cells armed with the control antibody CD18:gp55 developed tumors and died within 50 days following challenge with the parental hepa 1-6 cells (FIG.4). This experiment was repeated twice with comparable results.

EXAMPLE 6.6

APPLICATION OF THE METHOD OF THE INVENTION TO THE HEPATOMA CANCER SYSTEM: CURE OF ESTABLISHED HEPAOTOMAS WITH CYTOKINE TREATED, Bi-MAb-ARMED HEPA 1-6 CELLS

To establish that immunization with CT-hepa 1-6 cells armed with anti-CD28 Bi-MAbs can cure established hepatomas, the following three studies were performed, each of which indicates that the therapeutic administration of Bi-MAb-armed, cytokine treated hepa 1-6 cells is an effective therapy for hepatoma.

In the first study, forty mice were inoculated subcutaneously with 2 x 10^6 wild type hepa 1-6 cells. Fourteen days later, after the development of microscopic tumors, the mice were divided into four groups of ten each. The groups were treated subcutaneously with 2 x 10^6 cytokine treated or untreated hepa 1-6 cell armed with either the CD28:gp55 Bi-MAb or a control CD18:gp55 Bi-MAb.

The results of this study are shown in FIG. 5. All mice in the group treated with CT-Hepa 1-6 cells armed with anti-CD28 Bi-MAb survived for more than 100 days (FIG 5). In contrast, all mice in the group treated with control Bi-MAb-armed/cytokine

treated hepa 1-6 cells, and all mice in the groups treated with untreated hepa 1-6 cells armed with the CD28:gp55 Bi-MAb or the CD18:gp55 control Bi-MAb, died within about 40 days (FIG. 5). This experiment was repeated twice with comparable results.

In the second study, five groups of five mice each were injected subcutaneously with 1 x 10^6 hepa 1-6 cells. After four weeks, mice bearing tumors 6-8 mm (in the greatest dimension) were injected subcutaneously with 1 x 10^6 cytokine treated hepa 1-6 cells armed with the CD28:gp55, CD28:gp95 or CD28:gp210 Bi-MAbs, respectively, and then injected subcutaneously with a boost of the Bi-MAb-armed/cytokine treated hepa 1-6 cells at the same dose 7 days thereafter. Cells pre-incubated with CD18:gp55 or mixed with both anti-CD28 and anti-gp55 parental Mabs (CD28+gp55) at a concentration of 50 ug each were used as controls. Tumor size was periodically measured.

The results of this study are shown in FIG. 6. Hepatomas in the mice treated with the CD28:gp55, CD28:gp95 and CD28:gp210 Bi-MAbs regressed to undetectable size within about 40 days (FIG. 6). In contrast, hepatomas in the mice treated with the CT-hepa 1-6 cells pre-incubated with either control Bi-MAb or MAb more than doubled in size within the same period, and all of these mice within that period (FIG. 6). This experiment was repeated three times with comparable results.

In the third study, γ-irradiated Bi-MAb armed tumor cells were used as the vaccine. Three groups of five mice were inoculated subcutaneously with 1 x 10^6 hepa 1-6 cells. After two weeks, mice were then injected subcutaneously either with 1 x 10^6

γ-irradiated, cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb, or with a combination of γ-irradiated, cytokine treated hepa 1-6 cells plus a mixture of parental anti-CD28 and anti-gp55 Mabs, or with 1 x 10^6 γ-irradiated hepa 1-6 alone.

The results of this study, shown in FIG. 7, are similar to the results obtained in the second study described above. Only the mice treated with the γ-irradiated, cytokine treated hepa 1-6 cells armed with CD28:gp55 Bi-MAb survived (for more than 100 days). All mice in the other two groups died within 40 days of treatment.

Upon examination, tumor tissue from mice first injected with the parental tumor cells and then given cytokine treated autologous tumor cells pre-incubated with anti-CD28 Bi-MAb showed marked inflammatory responses with abundant lymphocyte infiltration. In accordance with in vitro stimulation data, the majority of the infiltrating lymphocytes were $CD3^+CD8^+$ $CD25^+$ T cells, as determined by immunofluorescent staining of tissue sections with rat anti-mouse CD3, CD8 and CD25 MAbs. There was no local immune response in mice injected either with untreated tumor cells armed with anti-CD28 Bi-MAb or with cytokine treated tumor cells armed with control Bi-MAbs.

To further investigate if the induced immunity was mediated by CTLs, mice were depleted of $CD8^+$ T cells by antibody treatment before or after immunization. Depletion of $CD8^+$ T cells either before or after immunization abrogated the ability of the cellular vaccine to elicit anti-tumor immunity in vivo.

EXAMPLE 6.7

APPLICATION OF THE METHOD OF THE INVENTION TO ADDITIONAL CANCER SYSTEMS: INDUCTION OF PROTECTIVE IMMUNITY AGAINST LYMPHOMA AND COLON CARCINOMA USING CYTOKINE TREATED, Bi-MAb-ARMED CANCER CELLS

The immunogenicity of cytokine treated, autologous tumor cells pre-incubated with anti-CD28 Bi-MAbs was tested in two additional cancer systems, EL-4 lymphoma and SMCC-1 colon carcinoma.

The EL-4 lymphoma becomes immunogenic when transfected with the B7 gene. In contrast, SMCC-1 colon carcinoma remains non-immunogenic even after transfection with the B7 gene. Both of these cell lines grow rapidly and develop subcutaneous tumors in syngenic C57 BL/6 mice (see, for example, Li et al., 1996, J. Exp. Med. 180: 211). Both of these cell lines express the gp55 antigen on their cell surfaces. Accordingly, the anti-CD28:gp55 Bi-MAb described in the previous examples was also used in these studies.

Three groups of mice were immunized subcutaneously with 1 x 10^6 cytokine treated, CD28:gp55 Bi-MAb-armed, hepa 1-6, EL-4 or SMCC-1 tumor cells respectively.

After two weeks, mice immunized with the modified hepa 1-6 cells were divided into three groups and challenged by a subcutaneous injection with 1 x 10^6 hepa 1-6 cells, SMCC-1 cells or EL-4 cells respectively.

The mice immunized with the cytokine treated, CD28:gp55 Bi-MAb-armed SMCC-1 cells were divided into two groups. One group was challenged by subcutaneous inoculation with 1 x 10^6 SMCC-1 cells. The other group was challenged by subcutaneous

inoculations of EL-4 cells and 1 x 10^6 hepa 1-6 cells into the left and right flanks of mice, respectively.

Similarly, the mice immunized with the cytokine treated, CD28:gp55 Bi-MAb-armed EL-4 cells were divided into two groups which were challenged with either EL-4 cells alone or with both SMCC-1 and hepa 1-6 cells.

The results of this study, presented in Table I below, were repeated twice with identical results.

TABLE I
Specificity of the immune responses elicited by cytokine treated and anti-CD28 Bi-MAb armed tumor cells.

IMMUNIZATION CELLS	CHALLENGE CELLS	NUMBER OF MICE WITH TUMORS
Bi-MAb-CT Hepa 1-6	Hepa 1-6	0 of 10
Bi-MAb-CT Hepa 1-6	SMCC-1	6 of 6
Bi-MAb-CT Hepa 1-6	EL-4	5 of 5
Bi-MAb-CT SMCC-1	SMCC-1	0 of 6
Bi-MAb-CT SMCC-1	EL-4 + Hepa 1-6	6 of 6
Bi-MAb-CT EL-4	EL-4	0 of 6
Bi-MAb-CT EL-4	SMCC-1 + Hepa 1-6	6 of 6

Immunization with cytokine treated EL-4 (CT-EL-4) or cytokine treated SMCC-1 (CT-SMCC-1) tumor cells armed with the anti-CD28:gp55 Bi-MAb elicited anti-tumor immunity against autologous parental tumor in all animals (Table 1).

The elicited immunity in all three experimental groups was tumor-specific. For example, immunization with CT-hepa 1-6 cells armed with anti-CD28 Bi-MAb did not inhibit growth of syngeneic

EL-4 or SMCC-1 tumors in vivo (Table 1). Interestingly, in the absence of treatment with cytokines, the parental EL-4 cells expressed high levels of MHC class I at cell surfaces, yet when pre-incubated with anti-CD28:gp55 Bi-MAb, they were still not able to induce protective immunity. In addition, CTLs from mice immunized with cytokine treated autologous tumor cells armed with anti-CD28 Bi-MAbs specifically lysed the parental tumor cells but not other tumor cells in vitro.

EXAMPLE 6.8

INDUCTION OF CELLULAR IMMUNITY BY TUMORS ARMED WITH MULTIVALENT BRIDGE MOLECULES

The above data showed that SMCC-1, EL-4 and Hepa1-6 cells became more immunogenic after treatment *in vitro* with a combination of cytokines and anti-CD28 bispecific monoclonal antibody. The modified tumor cells were able to elicit anti-tumor specific immunity that were both preventive and curative.

The following data showed that EL-4 and SMCC-1 cells were also effective for eliciting preventive and curative antitumor immunity in syngenic animals without cytokine treatment when these cells were precoated with two different bispecific monoclonal antibodies (bi-Mabs), one specific for CD28 and another specific for 4-1BB.

The cells were coated *in vitro* with anti-gp55:anti-CD28 and anti-gp115:anti-4-1BB bi-Mabs in a concentration of 50 ug/ml on ice for 45 min. After fixed with PEG and washed for three times,

the tumor cells coated with the two different bi-Mabs were subcutaneously injected into syngenic animals at different doses. Two weeks later, the immunized animals were challenged with parental tumor cells and tumor formation rate was observed (Table II).

Table II.Comparison of the efficacy of immunogenic tumor cells modified with different process *in vitro* on eliciting preventive antitumor immunity *in vivo*

Immunization	Dose	Challenge	Tumor Formation
Irradiated EL-4 Wt	$1x10^6$	$1x10^6$ EL-4 Wt	100%
Irradiated CT-EL-4Wt	$1x10^6$	$1x10^6$ EL-4 Wt	100%
Irradiated EL-4 coated CD28BiMab	$1x10^6$	$1x10^6$ EL-4 Wt	50%
Irradiated CT-EL-4 coated CD28BiMab	$1x10^6$	$1x10^6$ EL-4 Wt	0%
	$1x10^5$	$1x10^6$ EL-4 Wt	20%
	$5x10^4$	$1x10^6$ EL-4 Wt	60%
Irradiated EL-4 Wt coated anti-CD28&anti-4-1BB BiMabs	$1x10^6$	$1x10^6$ EL-4 Wt	0%
	$1x10^5$	$1x10^6$ EL-4 Wt	0%
	$5x10^4$	$1x10^6$ EL-4 Wt	10%

In curative experiments, syngenic animals were first inoculated subcutaneously with $2x10^6$ parental tumor cells. After two to four weeks, the tumor bearing animals were injected with the modified tumor cells. The mean survival time was monitored (Table III).

Table III. Comparison of the efficacy of immunogenic tumor vaccines armed with monovalent or multivalent bi-specific Mabs on eliciting curative antitumor immunity *in vivo*

Tumors	Vaccination	Dose	Animal Survival (%)*
SMCC-1	SMCC-1Wt	$1x10^6$	0%
SMCC-1	CT-SMCC-1+CD28 BiMabs	$1x10^6$	100%
		$5x10^5$	30%
SMCC-1	SMCC-1+CD28 & 4-1BB BiMabs	$1x10^6$	100%
		$5x10^5$	80%
SMCC-1	SMCC-1+CD28& 4-1BB BiMabs (multivalent BiMabs)	$1x10^6$	100%
		$5x10^5$	100%
		$1x10^5$	60%

* 60 day survival rate after tumor vaccine treatment.

EXAMPLE 6.9

GENERATION OF CELLULAR IMMUNITY AGAINST VIRUS INFECTED CELLS

Primary liver cells were obtained from clinical biopsy laboratory and cultured in hepatocellular media. Autologous peripheral blood lymphocytes were obtained from same patients during operation and cultured in complete RPMI-1640 medium supplemented with 5% human AB serum and 5% fetal calf serum, 20 iu/ml rh-IL-2.

Liver cells were infected by E1B deleted Adenovirus as reported previously. Virus infection was confirmed by RT-PCR and histological examination.

Infected liver cells were then treated *in vitro* with (1) cytokines alone, (2) BiMabs alone, or (3) cytokine + bispecific Mabs. The modified virus-infected liver cells were irradiated at

a dose of 5000R and were then co-cultured with autologous PBL in complete RPMI-1640 medium as reported previously.

The cytotoxicity of the CTLs generated by the unmodified and the modified liver cells was determined with a standard 4 h ^{51}Cr release assay.

Table IV.Generation of cytotoxic T cells *in vitro* by stimulating autologous peripheral blood lymphocytes with virus-infected human fetal liver cells pretreated with a combination of INF-γ and TNF-α and coated with anti-CD28 bispecific monoclonal antibody

Target cells	Treatment	Effectors	E:T Ratio	Cytotoxicity
Liver cells	None	PBL	1:50	~3%
Liver cells	Cytokines	PBL	1:50	~3%
Liver cells	BiMabs	PBL	1:50	~3%
Liver cells	Cyto+BiMab	PBL	1:50	~3%
Ad-Liver cells	None	PBL	1:50	~3%
Ad-Liver cells	Cytokines	PBL	1:50	~5%
Ad-Liver cells	BiMabs	PBL	1:50	~13%
Ad-Liver cells	Cyto+biMabs	PBL	1:50	~20%
Ad-Liver cells	biMabs (Multivalent*)	PBL	1:50	~40%
Ad-Liver cells	Cyto+biMabs(Multivalent*)	PBL	1:50	~40%

·Anti-Gp115:Anti-CD28 bispecific monoclonal antibody was generated by chemical linking several anti-CD28 and anti-Gp115 monoclonal antibodies together and purified by sequential affinity columns, one specific binding gp-115 and another for CD28 monoclonal antibody specifically.

The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single

illustrations of individual aspects of the invention, and any which are functionally equivalent are within the scope of the invention. Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description, and are similarly intended to fall within the scope of the invention. For example, Guo et al., Nature Medicine, vol. 4:1-5, (April, 1997) provide examples and references.

All publications referenced are incorporated by reference herein, including drawings, nucleic acid sequences and amino acid sequences listed in each publications. All the compounds disclosed and referred to in the publications mentioned above are incorporated by reference herein, including those compounds disclosed and referred to in articles cited by the publications mentioned above.

Other embodiments of this invention are disclosed in the following claims.